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SHORT COMMUNICATION

Diversity of Amylase-Producing *Bacillus* spp. from "Tape" (Fermented Cassava)

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Fermented cassava or "Tape" is one of traditional Indonesian fermented food. The quality of "Tape" is determined by microorganisms involved during fermentation process. It was reported that *Bacillus subtilis* determined the quality of cassava "Tape". The most common way to identify species is by using 16S rRNA gene. This gene contains conserved regions as unique sequence which is relative among species. It has been widely used as a reliable molecular marker for phylogeny identification. Therefore, the aim of this research was to study diversity of amylase-producing *Bacillus* spp. from "Tape" based on 16S rRNA gene sequences. *Bacillus* spp. were isolated from "Tape" from several area in Indonesia i.e. Jakarta, Bandung, Cianjur, Subang, Rangkas Bitung, and Kediri. Amplification of 16S rRNA gene used 63f and 1387r primers. This research showed that based on 16S rRNA gene sequences, twenty-six of amylase-producing *Bacillus* spp. isolates were divided into four groups. All isolates were identified as species either *B. megaterium, B. subtilis, B. amyloliquefaciens,* or *B. thuringiensis.*

Keywords: *Bacillus* sp., cassava fermentation, diversity, amylase

INTRODUCTION

Most people in the world consume fermented foods. Many fermented foods contain of ingredients that are good for health such as stimulating intestinal immunity and improving the balance of microbial population in gastrointestinal tract. Therefore, fermented foods will become even more important in our diet for maintaining the health (Farnworth 2003).

"Tape" is one of the famous fermented food from Indonesia. It is made from steamed cassava (*Manihot* utilissima), then mixed with starter commonly referred as "Ragi Tape" (Barus & Wijaya 2011). "Tape" is produced using traditional methods which have some drawbacks, such as not standardized manufacturing processes and the products (Pawiroharsono 2007). This could be resulted from the inconsistency of microbial composition in starter, also the influence of environmental factors. The quality of "Tape" depends on quality of cassava, preparation method, and microbes. Starter of "Tape" comprise a consortium of microbes consist of molds, yeasts and bacteria. These microbes will determine the quality of "Tape" due to their role during fermentation process.

The role of *Bacillus* spp. in improving quality of fermented food was reported, such as in Indian kinema (Sarkar *et al*. 2002), Korean cheonggukjang (Kwon *et al*. 2009), African dawadawa (Terlabie *et al*. 2006) and soumbala (Sarkar *et al*. 2002). It was also reported that *Bacillus subtilis* determined the quality of cassava "Tape" (Barus & Wijaya 2011). However, there is very limited of information on diversity of *Bacillus* strains present in conventional prepared "Tape".

A range of molecular biological approaches has been applied to study genetic diversity of microbes, predominantly based on the analysis of 16S rDNA genes. Therefore, this study aimed to study the diversity of amylase-producing *Bacillus* spp. from cassava "Tape" based on 16S rRNA gene sequences. The results will be used as basis for further analysis of the role of *Bacillus* strain in determining the quality of the "Tape".

MATERIALS AND METHODS

Isolation of *Bacillus* spp. Genome. Twenty-six of amylase-producing *Bacillus* spp. were isolated

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from "Tape". The samples of "Tape" were obtained from several area in Indonesia i.e. Pasar Bendungan Hilir-Jakarta, Pasar Kopro-Jakarta, Pasar Kelapa Gading-Jakarta, Pasar Petukangan-Jakarta, Pasar Kebon Jeruk-Jakarta, Bandung, Cianjur, Subang, Rangkas Bitung, and Kediri. Amylase activity test was done according to the method of Oguntoyinbo *et al.* (2006). Bacterial cultures were grown overnight at 30 °C in 50 ml of Luria-Bertani broth. Cells were recovered by centrifugation at 13,000 × g for 3 min. Cell pellet was resuspended in1 mL of 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS. Genomic DNA was isolated using *Fermentas® Genomic DNA Purification Kit* (Fermentas, Lithuania) based on the manufacturer's protocol.

Amplification and DNA Sequencing of 16S rRNA Gene. Amplification of 16S rRNA genes sequence of *Bacillus* spp. was performed in GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using the universal primers, comprises the forward primer 63F (5'-CAGGCCTAA CACATGCAAGTC-3') and the reverse primer 1387R (5'-CAGGCCTAACACATGCAAGTC- 3') (Marchesi *et al.* 1998). The primers were targeted to conserved regions and permitted the amplification of an aproximately 1,300 bp rDNA fragment. PCR master mix (50 µl) contained 25 µl *GoTaq Green* (Promega, Madison, USA), 17 µl *Nuclease Free Water* (Promega, Madison, USA), 2 µl of each primer, and 4 µl DNA template [± 100 ng]. PCR conditions were as follows: pre-denaturation at 95 °C for 5 minutes was followed by 30 cycle of denaturation at 95 °C for 1 minute, annealing at 58 °C for 5 minutes, extension at 72 °C for 1 minute, and post extension at 72 °C for 10 minutes. PCR products were observed using 1% electrophoresis agarose gel (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA). UV transilluminator was routinely used to visualize DNA in gel electrophoresis. PCR products were then partially sequenced in Macrogen Inc., Republic of Korea. The DNA sequencing results were aligned with 16S rRNA genes sequence database provided by GenBank (www.ncbi.nlm.nih.gov) using *Basic Local Alignment Search Tool* (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using MEGA 5 software (Tamura *et al*. 2011). Neighbour joining method was used to develop phylogenetic tree.

RESULTS

A total twenty-six of amylase-producing *Bacillus* spp. were obtained from "Tape". The *Bacillus* spp. have diverse amylase activities with range from 3.40

Table 1. Characteristics of amylase-producing *Bacillus* spp. from cassava "tape"

Sampel origin	Isolate code	Isolate code/homology	Maximum identity(%)	Accession number	Amylase activity (units/ml)
Bandung	Bdg 1-163f	*B. amyloliquefaciens* strain SWI4b	99	JX203228.1	24.18
	Bdg 1-2163f	*B. amyloliquefaciens* strain BVC13	96	JQ660596.1	22.36
Cianjur	Cjr 1163f	*B.amyloliquefaciens strain NBRC 15535*	99	NR_041455	28.55
	Cjr 4163f	*B. amyloliquefaciens* isolate 003114	98	NR_027552	11.36
	Cjr 5163f	*B. subtilis* strain M34	96	HQ204325.1	31.04
Jakarta Timur	Pkg 2163f	*B. thuringiensis* strain Bi51	97	HQ336298.1	23.57
	Pkg 3163f	*B.thuringiensis strain IAM 12077*	98	NR_043403	16.28
	Pkg 4163f	*B. subtilis subsp. subtilis strain DSM 10*	100	NR_027552	24.97
	Pkj 2163f	*B.subtilis* strain CCGE2066	96	EU867368.1	21.63
	Pkj 3163f	*B. subtilis subsp. subtilis strain DSM 10*	98	NR_027552	24.48
Jakarta Pusat	Psb 1-1163f	*B. megaterium strain IAM 13418*	99	NR_043401	29.16
	Psb 1-2163f	*B.* amyloliquefaciens strain W49	99	KC441855.1	14.70
	Psb 2-1163f	*B.amyloliquefaciens* strain W49	99	KC441855.1	4.80
Jakarta Barat	Psk 1163f	*B.megaterium strain IAM 13418*	99	NR_043401	15.19
	Psk 3163f	*B. megaterium strain IAM 13418*	97	NR_043401	27.34
	Psk 4163f	*B. megaterium strain IAM 13418*	99	NR_043401	32.02
	Ptk 3163f	*B. subtilis subsp. subtilis strain DSM 10*	98	NR_027552	24.00
Rangkas Bitung	Rbt 2163f	*B. megaterium strain IAM 13418*	96	NR_043401	35.97
	Rbt 3163f	*B. subtilis subsp. subtilis strain DSM 10*	99	NR_027552	32.93
Subang	Sbg 1163f	*B. megaterium strain IAM 13418*	96	NR_043401	11.18
	Sbg 3163f	*B. amyloliquefaciens* strain W49	98	KC441855.1	6.14
Kediri	Smk 1163f	*B. amyloliquefaciens strain NBRC 15535*	99	NR_041455	4.62
	Smk 3163f	*B. amyloliquefaciens strain NBRC 15535*	100	NR_041455	29.10
	Smk 4163f	*B. amyloliquefaciens strain NBRC 15535*	99	NR_041455	3.16
	Smk 5163f	*B.subtilis subsp. subtilis strain DSM 10*	99	NR_027552	7.17
	Smk 8163f	*B.amyloliquefaciens strain NBRC 15535*	98	NR_041455	3.40

to 35.97 unit/ml at 37 °C (Table 1). Genome of all *Bacillus* spp. isolates have been successfully isolated from cell cultures using protocol kit of Fermentas® Genomic DNA Purification Kit. PCR amplification of 16S rRNA gene sequences yielded DNA fragments with single band at 1,300 bp for each *Bacillus* sp. strains (data not shown).

BLASTN results of the partial sequence of 16S rRNA gene (about 800 to 950 nucleotides) showed high similarity with *Bacillus* spp. with maximum identities for each isolate in range of 94-100% with E-value 0 (Table 1). The distribution of all isolates was only on four species of *Bacillus* sp. such as *B. megaterium*, *B. subtilis*, *B. amyloliquefaciens*, and *B. thuringiensis*.

Neighbour joining tree based on 16S rRNA gene sequences was successfully constructed. Phylogenetic tree showed the relation among twenty-six of *Bacillus* sp. isolates (Figure 1). It showed that the isolates were divided into four groups (Figure 1). Group 1 (11 isolates), group 2 (seven isolates), group 3 (two isolates), and group 4 (six isolates) were quite related to *B. amyloliquefaciens*, *B. subtilis*, *B. thuringiensis*, and *B. megaterium* references strains respectively (Tabel 1).

DISCUSSION

This is a preliminary study of amylase-producing *Bacillus* spp. from "Tape". A total nine samples of "Tape" examined contained of amylase-producing *Bacillus* spp. *Bacillus* spp. are the organisms which responsible for any food fermentations and spoilage of foods in general, due to their versatile metabolism and heat resistant spores. Several fermented products rely on the participation of various *Bacillus* species,

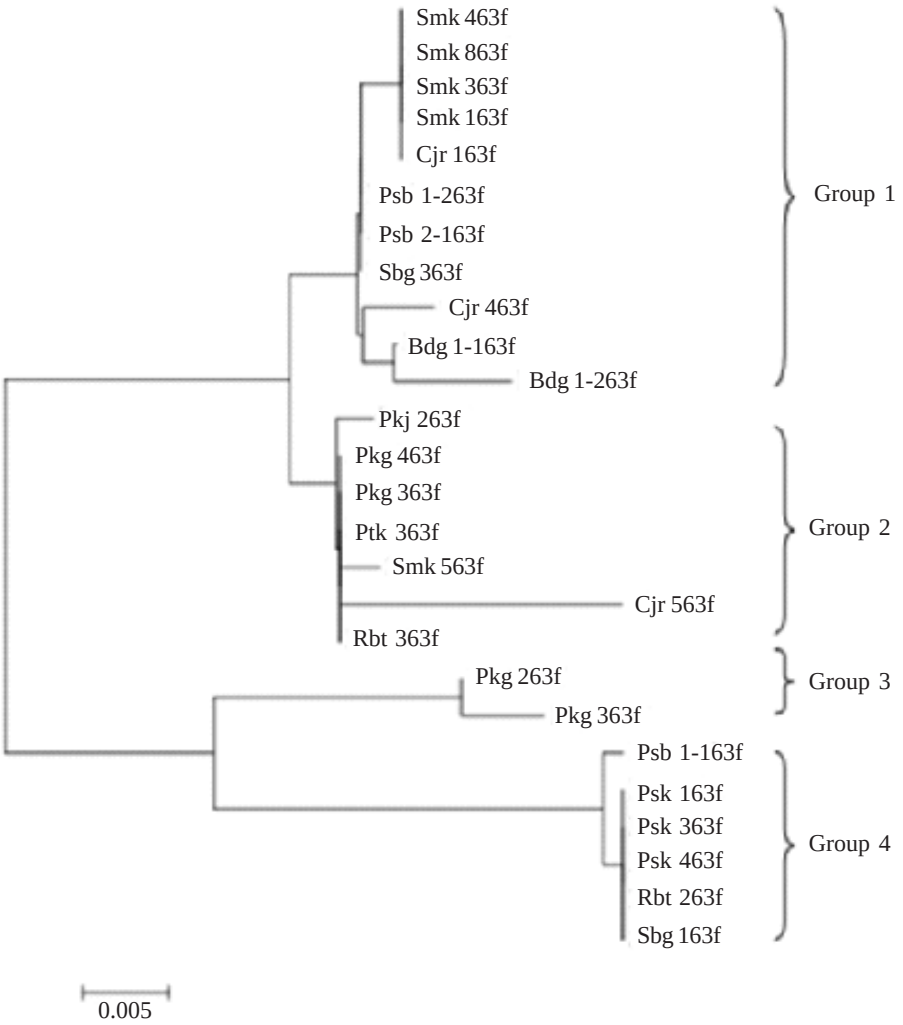

Figure 1. Dendogram showing genetic diversity among the partial 16S rDNA sequence of 26 amylase - producing *Bacillus* spp. strains from cassava fermented "Tape". The neighbour joining (NJ) tree was constructed using Mega 5 with 100 bootstrap analysis of replication.

including cassava fermented. It is widely distributed in many country such as: *B. cereus* and *B. subtilis* in Lafun – African (Padonou *et al*. 2009), *Bacillus* spp. in ‘‘attie´ke´’’ – Abidjan (Assanvo *et al*. 2006), *Bacillus* sp. in “fufu” – Nigeria (Achi & Akomas 2006), and *B. subtilis* in akyeke – Ghana (Obilie *et al*. 2003).

Bacillus spp. have an important role in cassava fermentation. During fermentation, many *Bacillus* produce enzymes, which are hydrolyzed oligosaccharides into easily digestible sugars (Chantawannakul *et al*. 2002; Joo *et al*. 2007). *Bacillus* spp. influenced the taste and aroma of “fufu” (Okafor *et al*. 2008). *Bacillus* sp. was also reported involve in the textural modification of cassava tissue during fermentation (Amoa-Awua & Jakobsen 1995; Obilie *et al*. 2003). It was reported that *B. subtilis* produced flavors of “Tape” most preferred by panelists (Barus & Wijaya 2011).

The results of this study showed that there were *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium*, and *B. Thuringiensis* found in “Tape”. Amylase assay (Table 1) showed that all *Bacillus* spp. isolates produced diverse amylase activity. *Bacillus* spp. isolates in each group also have diverse amylase activities. Example, group 1 (11 isolates) have amylase activities with range from 4.80 to 29.10 unit/ml at 37 °C (Table 1). The Rbt 2, Rbt 3, and Smk 3 isolates that had highest amylase activity were closely related to *B. megaterium*, *B. subtilis*, and *B. amyloliquefaciens* respectively. The isolates will be studied further to determine their amylase activity and role in improving qualty and taste of tape.

Phylogenetic dendogram based on 16S rRNA gene sequences (Figure 1) generated four groups. The groups were dominated by *B. amyloliquefaciens*, *B. subtilis*, *B. thuringinensis*, and *B. megaterium* (Table 1). The isolates in the same group were closely related to one reference species of *Bacillus* with maximum identities in range of 94-100%.

In this study, sequences of the 16S rDNA can be used to reveal diversity of *Bacillus* spp. accordance with those reported by Wahyudi *et al*. (2010)

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